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Spontaneous Mutations of Trichlorfon Resistance in the Nematode, *Caenorhabditis elegans*

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ABSTRACT—Spontaneous trichlorfon-resistant mutations were isolated in *Caenorhabditis elegans* var. Bergerac and its derived mutator strains. Of these, six uncoordinated mutations were assigned by complementation analyses to the *unc-13*(*cn490*), *unc-17*(*cn355*), *unc-18*(*cn347*), *unc-10*(*cn257*), *unc-3*(*cn4146*) and *unc-41*(*cn252*) genes. Resistance of these mutations to acetylcholinesterase (AChE) inhibitors is partial, with the extent dependent on the mutations. These mutations fall into two classes based on acetylcholine (ACh) levels, that is, the normal ACh levels in *unc-10* and *unc-3* mutations and the abnormally high ACh levels in *unc-13, unc-17, unc-41* and *unc-18* mutations. Mutations in the latter gene groups are also accompanied by growth retardation and small body size in adulthood. Double mutants were constructed between the six trichlorfon-resistant strains together with the *cha-1·unc-17* complex gene alleles which are also resistant to trichlorfon. All doubles constructed between trichlorfon-resistant strains survived and their properties were studied.

INTRODUCTION

The presynaptic terminal liberates a neurotransmitter in response to depolarization [1]. However, little is known about the mechanism underlying the synthesis, storage and release of neurotransmitters. Acetylcholine (ACh) is one of the primary neurotransmitters. Although there are extensive studies on the synthetic enzyme choline acetyltransferase (ChAT) and the receptor functions of cholinergic neurons [1–3], there are few genetic studies on the profiles of ACh at the presynaptic terminal. The nematode *C. elegans* has several advantages as an exprimental organism for genetic manipulations [4].

Johnson and Stretton [5] identified inhibitory and excitatory classes of ventral cord motoneurons in *Ascaris* and pointed out the possibility that ACh is the neurotransmitter used by the excitatory motoneurons. Because of its morphological similarity, ACh appears to be a neurotransmitter in the ventral cord neurons of *C. elegans* [6]. The complex gene *cha-1·unc-17* consists of at least two parts, the *cha-1* region and the *uch-17* region. Although the *cha-1* encodes the structural gene for ChAT [7–9], the function of the *unc-17* region is obscure. Although ACh levels in the *cha-1* mutations decreased and were accompanied by a reduction in ChAT activity, ACh levels were abnormally high in the *unc-17* mutation in spite of normal ChAT activity [10]. We demonstrated the possibility that ACh of the mutant accumulates at the presynaptic terminal rather than at the synaptic gap. To identify additional genes that affect profiles of ACh, were carried out a further isolation of mutants. Alleles of the *cha-1·unc-17* complex gene are resistant to inhibitors of acetylcholinesterase (AChE). Therefore, we isolated resistants to trichlorfon, one of the potent AChE inhibitors.

For mutant screening, *C. elegans* var. Bergerac strain BO and its derived mutator strains were used instead of the var. Bristol strain N2 which has been well- characterized. BO and mutator strains produce spontaneous mutations at a high rate [11]. Many of these mutations appear to be brought about by the insertion of transposable element Tcl into the gene which makes it possible to tag the gene with Tcl as a probe [12, 13].

In *C. elegans*, the transposable element Tcl was identified as a repetitive sequence [14–17]. It was found that in the *unc-22* [18] and *unc-54* genes [19,

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20], the frequency of spontaneous mutation caused by Tcl transposition was much higher in the BO than in the N2 strain. Tcl has now become an useful tool for cloning *C. elegans* genes identified by insertional mutation as already successfully shown in the genes *unc-22* [13] and *lin-12* [12].

In an attempt to clone genes in the future, we have isolated the spontaneous mutants resistant to trichlorfon with BO and its derived mutator strains. Of the trichlorfon resistants isolated, the properties of six mutants are presented here.

MATERIALS AND METHODS

General handling Culturing, stock maintenance and genetic manipulation of *C. elegans* were performed as described by Brenner [4]. All experiments were carried out at 20°C unless otherwise noted.

Strains, genes, and alleles of C. elegans The gene alleles used are listed below by linkage groups. Positions of these genes on the *C. elegans* genetic map are shown in Figure 1. Standard nomenclature for *C. elegans* genotypes and phenotypes is used according Horvitz *et al.* [21].

LGI: *unc-13*(*cn490, e1019*), *dpy-5*(*e61*), *unc-63*(*e384*), *unc-11*(*e47*), *lin-6*(*e1416*), *unc-35*(*e259*).

LGII: *dpy-10*(*jk64*)

LGIII: *dpy-18*(*e364*), *unc-32*(*e189*), *unc-36*(*e251*), *unc-64*(*e246*)

LGIV: *cha-1*(*p1152, cn101*), *unc-17*(*e113, e245, cn355*), *unc-33*(*e204*), *dpy-13*(*e184*), *dpy-20*(*jk142*), *lin-1*(*e275*).

LGV: *dpy-11*(*e224*), *unc-42*(*e270*), *unc-23*(*e25*) *unc-41*(*e268, cn252*), *rol-3*(*e754*), *sma-1*(*e30*), *unc-65*(*e351*).

LGX: *unc-18*(*e81, cn347, md118, md120, md183, md193*), *unc-10*(*cn257, e1021*), *unc-3*(*cn4146, e151*), *lon-2*(*e678*), *dpy-6*(*e14*), *dpy-7*(*e88*), *mah-2*(*cn110*), *dpy-3*(*e27*), *unc-6*(*e78*), *unc-1*(*e74, e1598*), *unc-7*(*e5*)

Genetic properties of other strains used for three-factor crosses and the analysis of a Tcl polymorphism associated with the *unc-18*(*cn347*) mutation are summarized in Table 1.

Isolation of trichlorfon-resistant mutants The *C. elegans* var. Bergerac strain BO and its derived mutator strains RW7097(*mut-6*) and RW7464 (*mut-5*) were used for the resistant mutant isolation. The mutator strains RW7097 and RW7464 were generated from mutator/N2 hybrids as a Tcl-transposing strain carrying only about 60 copies of Tcl(I. Mori, personal communication).

Test animals were cultured at 16°C on 10 cm-diameter NGM plates and were washed off plates with M9 buffer immediately before food became exhausted. Collected animals were transferred to 5 cm-diameter NGM plates containing 0.1 mM trichlorfon and kept for 7 to 10 days. Animals that survived were picked up. Finally, one animal per test plate was selected as potentially resistant. The isolates were made congenic by 10 cycles of outcrossing with Bristol N2.

Drug sensitivity test Sensitivity of the animals to cholinergic reagents was tested in S medium or on NGM. Drugs were previously sterilized by filtration through 0.22 μm nitrocellulose filters. Behavioral analyses were mainly performed in a liquid medium while the survival test was on solid agar.

Preparation and assay of ChAT The method for the preparation and the determination of *C. elegans* ChAT has already been described else-

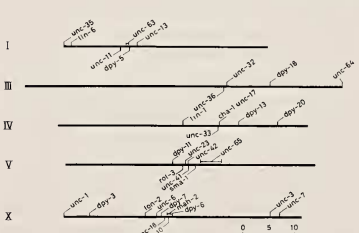

Fig. 1. Partial genetic maps of LGI, LGIII, LGIV, LGV and LGX showing locations of the trichlorfon-resistant gene and some of the other markers used in this study.

Abbreviations: unc, uncoordinated[4]; sma, small[4]; dpy, dumpy[4]; rol, roller[4]; lon, long[4]; mah, mahi[8]; cha, choline acetyltransferase[7].

TABLE 1. *C. elegans* strains used for a Tcl polymorphism associated with the *unc-18(cn347)* mutation

Strain	Genotype	Comments
TN347	*unc-18(cn347)*	Stable mutation
Tn3472	*unc-18(+)*	Intragenic revertant from TN347
TN3475	*unc-18(+)*	Intragenic revertant from TN347
TN1301	*unc-18(cn347)mah-2(cn110)*	Recombinant replaced DNA to the right of *unc-18*
TN1302	*mah-2(cn110)*	Recombinant replaced DNA to the left of *unc-18*
TN1303	*dpy-7(e88)*	Recombinant replaced DNA to the right of *unc-18*

All strains are primarily Bristol in chromosomal background.

where [7, 9, 22]. The procedures are briefly summarized as follows. Animals were washed extensively to remove bacterial contamination and suspended in extract buffer consisting of 100 mM Tricine (pH 8.0), 10 mM sodium thioglycollate, 1 mM phenanthroline, 1 mM EDTA, and 0.5 mM phenylmethyl-sulfonyl fluoride. The animals were ground to a fine powder in liquid nitrogen. After thawing, the preparation was used as a crude enzyme. The reaction mixture contained 50 mM Tricine (pH 8.0), 20 μM neostigmine bromide, 0.1 mM dithiothreitol, 1 mM EDTA, 0.5 mM acetyl-CoA, 30 μM choline, and 0.32 μCi [methyl-^{3}H]-choline chloride. The reaction was initiated by the addition of the enzyme and incubated at 5°C for 40 to 60 min. Protein concentration was detemined by using Coomassie Blue dye reagent with bovine serum albumin as a standard.

Choline and ACh levels The extraction and radiochemical assay of *C. elegans* choline and acetylcholine has been described previously [10, 23]. Briefly, the animals were lysed in a 1N-formic acid-acetone mixture, centrifuged at 1,000×g for 5 min whereupon the supernatant was evaporated to dryness under a stream of dry nitrogen. Dried samples were reconstituted with 0.1 N HCl. A 50 μl portion of the sample was mixed with 60 μl sodium tetraphenylboron. The organic phase was mixed with an equal volume of 0.4 M HCl and dried. Choline and ACh levels were measured radiometrically by the enzymatic conversion of choline to phosphoryl-choline [23]. For choline and ACh assays, nematodes were grown on 10 cm-diameter petri dishes which contained the same components as the NGM agar but with more bactopeptone (25 g/l). The dishes were allowed to grow about 0.2 g of *C. elegans*. Usually two dishes were used for one assay.

Southern blot hybridization The method for DNA extraction from nematodes has been described [24, 25]. Total genomic DNAs were digested with restriction enzymes and then electrophoresed on 1% agarose gels. Filter-transfer hybridizations were carried out by the methods of Southern [26]. A radiolabeled Tcl probe was prepared by nick translation [27].

Constructions of double mutants of trichlorfon resistant Double mutants were generated by a pairing combination of the following trichlorfon resistants: *unc-13(cn490)*, *unc-41(cn252)*, *cha-1·unc-17(p1152, cn101, e113, e245, cn355)*, *unc-10(cn257)*, *unc-18(cn347, e81)* and *unc-3(cn4146)*. The Dpy mutation belonging to the same linkage group of either one of two trichlorfon-resistant genes was accompanied by the doubles as visible phenotypes. The construction of *dpy-13(e184) unc-17(e245) unc-18(cn347) is described, as an example. N2 males were mated with dpy-13(e184) unc-17(e245)* hermaphrodites. The *dpy-13(e184)* mutation is semi-dominant, showing semi-Dpy phenotype in a *dpy-13/+* heterozygote. Semi-Dpy male progeny (*dpy-13·unc-17/+*) were mated with *unc-18(cn347)* hermaphrodites. Semi-Dpy hermaphrodites (*dpy-13·unc-17/+*; *+/unc-18*) were picked and the resultant Dpy-Unc progeny were randomly transferred to forty individual NGM

plates. The presence of the *unc-17* and *unc-18* mutations in the double mutants were tested by mating the Dpy-Unc hermaphrodites with *dpy-13(e184)·unc-17(e245)*/+ males. If the double was viable, both Dpy·Unc and semi-Dpy·Unc males appeared on the same plates. If the double was undetectable, the experiments were repeated twice. If no double was detectable on 120 NGM plates, the double was concluded to be lethal.

Chemicals Trichlorfon [(2, 2, 2, -trichloro-1-hydroxyethyl) phosphonic acid dimethyl ester], levamisole [L-[-]2, 3, 5, 6-tetrahydro-6-phenylimidazo[2, 1-b] thiazole], choline kinase (yeast) and AChE (electric eel, type V1-S) were purchased from Sigma. Aldicarb [2-methyl-2-(methylthio) propionaldehyde-O-(methylcarbamoyl) oxime] was provided by Union Carbide. [α-^{32}P] dCTP (3000 Ci/mmol), nick translation system and (methyl-^{3}H] choline chloride (180 Ci/nmole) were from Amersham. [γ-^{32}P] ATP (5209 Ci/nmol) was obtained from ICN Radiochemicals.

RESULTS

Effects of trichlorfon on C. elegans Prior to the resistant selection, the effects of trichlorfon on *C. elegans* were studied. Fourth larval (L4) animals were placed into S medium containing trichlorfon. Animals were not paralyzed instantaneously and it took several minutes to visualize abnormal phenotypes. L4 animals stopped their movement at around ten minutes in the presence of 10 mM trichlorfon but kept moving more than one hour in 1 mM of trichlorfon. The body of the paralyzed nimals shrunk so much that the mouth part was often extruded. Animals exposed for one hour to 10 mM trichlorfon could recover gradually from the paralysis upon withdrawal of the drug. The recovered animals laid eggs and were indistinguishable from nontreated animals in morphology and movement.

When newly-hatched larvae were placed on plates of growth medium containing trichlorfon,

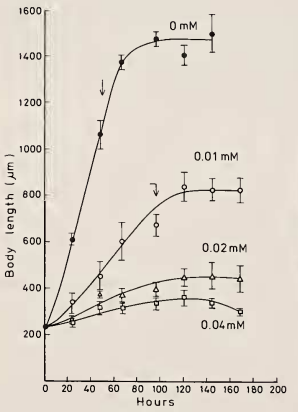

Figure 2 (A)

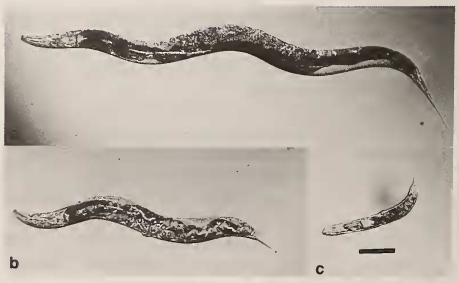

Figure 2 (B)

Fig. 2. Growth (A) and photographs (B) of hermaphrodites of *C. elegans* strain N2 grown in the presence of trichlorfon.
(A) Animals were grown on NGM at the indicated concentrations of trichlorfon.
S.D. is indicated by the vertical bar. Arrows indicate the time at which eggs were detected on test plates.
(B) Light micrographs of adult hermaphrodites cultured with 0 mM (a), 0.02 mM (b) and 0.04 mM (c) trichlorfon. Bar=0.1 mm.

the growth of the animals was impaired according to levels of drug concentration. Although animals were able to grow slowly on NGM containing 0.01 mM trichlorfon, they were small in body size at adulthood (Fig. 2). Some animals produced a very small number of progeny in the presence of 0.02 mM but not 0.04 mM of trichlorfon.

Isolation of trichlorfon resistants

Trichlorfon-induced paralysis provides a convenient basis for the isolation of resistant mutants. We carried out a screening of spontaneous mutations of trichlorfon resistance with *C. elegans* var. Bergerac (strain BO) and the Bergerac-derived mutator strain RW7097 and RW7464. Each strain was cultured on 150 plates of 10 cm-diameter NGM, transferred to test plates containing trichlorfon and animals which survived were picked as decribed in MATERIALS AND METHODS. We obtained two resistant strains from BO, three resistants from RW7097, and two resistants from RW7464. All resistants were accompained by uncoordinated phenotypes that were not segregated by extensive genetic studies.

Complementation tests and mapping Each mutation was assigned to a linkage group and the approximate locus was determined by a two-factor cross. Complementation tests were performed among uncoordinated mutations located near the locus on the same linkage group. Five mutations, *cn355, cn490, cn252, cn347, cn4146,* and *cn257* were assigned to genes that had been identified previously as *unc-17, unc-13, unc-41, unc-3,* and *unc-10,* respectively (Fig. 1), because no complementation was observed with the known mutant alleles at the respective gene. From the two- and three-factor crosses, one mutation *cn347* showed a tight linkage to *unc-18* (data not presented). Phenotypes of *cn347* and all the known mutations at the *unc-18* locus (*e81, md118, md120, md180, md193*) showed characteristics which were very similar to each other, i.e., kinky paralysis, slow growth, small body size in adulthood, and resistance to inhibitors of acetylcholinesterase. From these results, *cn347* is likely to be an allele of the *unc-18* gene. Complementation tests with *tra-1* males are in progress.

Evidence that the spontaneous mutants are induced by the insertion of Tcl Each of the six mutants was grown on 100 plates of 10 cm-diameter NGM and inspected for the appearance of revertant strains showing the wild-type phenotype in movement. Wild-type revertants were obtained from six plates of *cn252* animals, four plates of *cn257* animals and forty-one plates of *cn347* animals. From the plates of *cn490, cn355* and *cn4146,* no revertant was found. These results suggest that at least three mutations are induced by the insertion of a transposable element.

To prove directly that the spontaneous mutations are associated with Tcl insertion, we examined Tcl polymorphisms of the *unc-18*(*cn347*) mutation (Fig. 3). Southern blot analyses revealed that DNA from the TN347 strain had a novel 6.8 kb Tcl-containing DNA fragment that was not seen in DNAs from the RW7097 and N2 strains. Two independent non- *unc-18* revertants of the *cn347* mutation did not have the band suggesting that Tcl inserted into the *unc-18* gene caused the *cn347* mutation. Furthermre, the *unc-18 mah-2* recombinant (TN1301) chromosome showed the 6.8 kb band while the *dpy-7* non-*unc-18* recombinant (TN1303) chromosomes and *mah-2* non-*unc-18* recombinant (TN1302) chromosomes did not. This also suggests that the novel 6.8 kb Tcl band is mapped between *dpy-7* and *mah-2.*

General properties of trichlorfon resistants

The morphology, movement, and touch response of the six isolated mutants are described. Typical body forms of the adult hermaphrodite are shown in Fig. 4. Body forms of the *unc-10*(*cn257*) and *unc-3*(*cn4146*) are fat but correspond to the wild type in body size. However, body sizes of the other four mutants are smaller. All resistant strains are able to lay eggs. Body shapes are clearly different from animals bearing mutations in muscle genes such as *unc-52* or *unc-54* whose body is relaxed and limp. Bodies of the resistnants are kinky or coiled. A brief phenotypic description of the uncoordinated mutants has appeared elsewhere [28].

unc-13(*cn4146*): The animals are shrunken and have a fat body. The animals are defective in locomotion but respond with a slight movement of the head when the body is touched.

unc-17(*cn355*): The body of the mutant is thin and small as in the *unc-18*(*cn347*) mutation. The

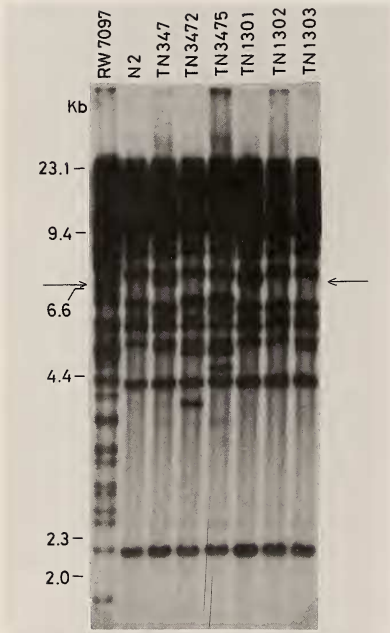

Fig. 3. A TcI polymorphism associated with the *unc-18*(*cn347*) mutation. Strain TN347 contains the spontaneous *unc-18*(*cn347*) mutation crossed ten times into Bristol N2. Strains TN3472 and TN3475 are spontaneous wild-type revertants of *cn347*. Strain TN301 is a recombinant containing the *unc-18*(*cn347*) *mah-2*(*cn110*) mutations. TN1302 is a recombinant containing the *mah-2*(*cn110*) mutation that replaced DNA to the left of *unc-18*. TN1303 is a recombinant containing the *dpy-7*(*e88*) mutation that replaced DNA to the right of *unc-18*. DNAs from these strains were digested with BglII and analyzed as described in MATERIALS AND METHODS. Animals with the spontaneous *unc-18* mutation *cn347* have a unique band of approximately 6.8 kb as indicated by arrows. The plasmid pCe2002, which contains TcI, was used as a hybridization probe. Relevant λ HindIII size standards (kb) are given to the left of the figure.

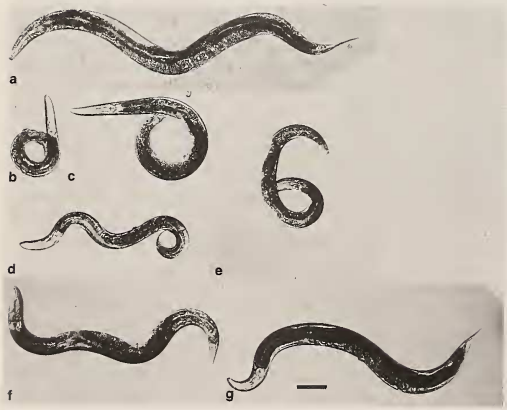

Fig. 4. Photomicrographs of adult hermaphrodites. Wild type (a), *unc-13*(*cn490*)(b), *unc-17* (*cn355*)(c), *unc-41*(*cn252*)(d), *unc-18*(*cn347*)(e), *unc-10* (*cn257*)(f), and *unc-3*(*cn4146*) (g). Bar = 0.1 mm.

body is shrunken and often coil. The animals are able to locomote rather smoothly. Although the animals escape rapidly when touched on the tail region, the animals coiled up their bodies when their heads are stimulated.

unc-41(*cn252*): The animal bodies are thin and shrunken. The animals smoothly locomote forward and are able to move backward by a repeating pause and movement when stimulated on their heads.

unc-18(*cn347*): The body of the mutant is thin and small. The animals are so severely paralyzed that locomotion is defective. When touched on the body, the animal is almost totally unresponsive except for a slight movement of the head.

unc-10(*cn257*): The animals are weakly coiled but move forward smoothly. The animals move backward by sinusoiding their bodies steeply when stimulated on the head.

unc-3(*cn4146*): The animals are fat but normal in body size. Although the body is almost paralyzed, the animals can locomote forward.

Developmental growth The growth rate of the six mutants was compared (Fig. 5). All the mutants are smaller in adult body size than the wild type. The difference in body size of the remaining four mutations *unc-17*(*cn355*), *unc-13*(*cn490*), *unc-41*(*cn252*) and *unc-18*(*cn347*) are quite marked. As shown by the arrows in Figure 5, mutants containing *cn257* and *cn4146* start egg-laying at around 50 hours after hatching, corresponding to the wild-type animals. On the other hand, the egg-laying of the other four mutants is remarkably retarded though the extent is variable.

Sensitivity to cholinergic reagents Survival of the six mutants was tested in the presence of cholinergic reagents that cause nematocide and were originally used as an anthelmintic (Table 2). The antagonist, decamethonium did not inhibit nematode growth. No mutant was resistant to the cholinergic anogist, levamisole compared to the wild-type animals. Trichlorfon, neostigmine, eser-

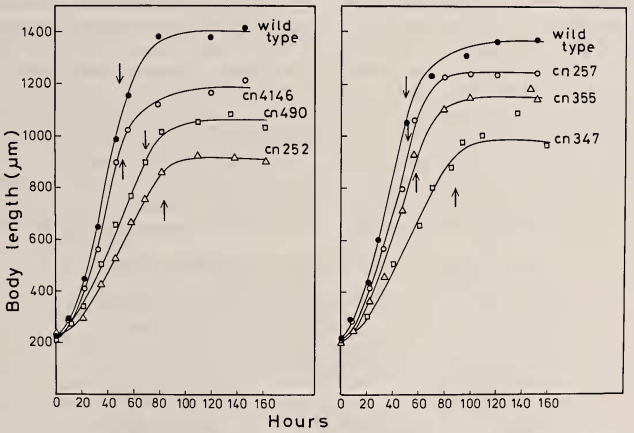

FIG. 5. Growth and egg-laying stage of each strain was prepared so that all worms hatched within a two-hour period. Worms were grown on NGM and at intervals, were suspended in one drop of M9 buffer. On a slideglass, worms were heated slightly and body length was measured with an eyepiece graticule. The mean value of seven worms is presented, but the bar for standard deviation is omitted. To determine the egg-laying stage, L4 larvae were transferred to 21 individual plates and inspected at two-hour intervals. The mean time is indicated by an arrow.

Table 2. The effect of cholinergic reagents on the growth of trichlorfon-resistant

Genotype	Trichlorfon mM	Growth on Aldicarb mM	Levamisole mM
Wild type	0.02	0.20	>0.30
unc-13(*cn490*)	0.10	>1.00	0.04
unc-17(*cn355*)	>0.30	>1.00	>0.30
unc-41(*cn252*)	0.20	>1.00	0.01
unc-18(*cn347*)	0.10	>1.00	0.01
unc-10(*cn257*)	0.10	>1.00	0.20
unc-3(*cn4146*)	0.04	0.80	>0.30

Three larvae were put onto NGM containing ten different concentrations of reagents (0, 0.01, 0.02, 0.04, 0.06, 0.08, 0.1, 0.15, 0.2 and 0.3 mM). Four different concentrations were further tested in the case of aldicarb at which the worm, in triplicate experiments, was able to produce F2 progeny within ten days.

ine, diisofluorophosphate, and aldicarb (2-methyl-2-(methoylthio) propionaldehyde-0-(methylcarbamoyl) oxine) are representative inhibitors to *C. elegans* AChE [29]. However, the extent of the effect on nematode growth is not always comparable to the degree of AChE inhibition. For example, 2 mM neostigmine and eserine did not influence nematode growth though they are potent inhibitors to AChE. At higher concentrations, the reagents caused *C. elegans* paralysis [8] and probably would inhibit growth. Of the mutants, the *cn355* animals were the strongest and the *cn4146* animals were the weakest in resistance to trichlorfon. The wild type animals were the most sensitive to aldicarb while the *cn4146* animals showed low significant resistance. The growth of *cn355* animals is not influenced by 1 mM aldicarb. Other mutant strains are also able to make F2 progeny but their growth was greatly suppressed at a high concentration of aldicarb. Although aldicarb is a much weaker inhibitor of nematode growth than trichlorfon, the mutant show a pattern of sensitivity to aldicarb which is almost the same as that observed for trichlorfon. Therefore, the mutants were not specifically resistant to trichlorfon but probably resistant to AChE inhibitors.

ChAT activity, choline and ACh levels

Mutant alleles of the *cha-1 unc-17* complex gene were all resistant to AChE inhibitors. However, these alleles were greatly different each other in ChAT activity and ACh levels [10]. Accordingly, ChAT activity, choline and ACh levels of the six mutants were measured (Table 3). Choline levels were all normal (data not shown). ChAT activity

Table 3. ChAT activity and Ach levels in the trichlorfon-resistant stains

Genotype	ChAT activity (μmol/h/mg protein)	ACh (nmol/mg protein)
Wild type	0.021±0.004	0.118±0.025
unc-13(*cn490*)	0.027±0.006	0.375±0.027
unc-17(*cn355*)	0.079±0.002	0.475±0.058
unc-41(*cn252*)	0.035±0.006	0.409±0.016
unc-18(*cn347*)	0.031±0.000	0.673±0.122
unc-10(*cn257*)	0.019±0.002	0.219±0.008
unc-3(*cn4146*)	0.023±0.002	0.119±0.026

Animals were grown at 20°C. Each value represents the mean±S.D. of three independent measurements of ChAT activity and of assays for ACh levels repeated three times.

was all within normal values, though the ChAT activity of *cn355* was about two times higher than that of the wild type as already reported with the other *unc-17* allele *e245* [10]. ACh levels of the remaining four mutants were abnormally high. All mutations showing high ACh levels were accompanied by abnormal development (Table 3 and Fig. 5). In the *unc-18* mutation, ACh levels are the highest and the developmental rate is the slowest of the six mutations. However, the extent of the retardation is different, irrespective of similar ACh levels in the *unc-17* and the *unc-41* mutations. A correlation between ACh levels and the extent of trichlorfon-resistance was not observed.

Double mutants between trichlorfon resistants

In order to test the interaction between the trichlorfon resistant mutations, we tried to construct double mutants of following mutations on different linkage groups: *unc-13*(*cn490*), *cha-1*(*p1152, cn101*), *unc-17*(*e113, e245, cn355*), *unc-41*(*cn252*), *unc-10*(*cn257*), *unc-18*(*cn347, e81*) and *unc-3*(*cn4146*). All possible combinations of doubles between them were detectable. However, the growth was poor in all the double mutants, especially in the *unc-17*·*unc-18* double mutations. To determine which gene was expressed in the doubles, ACh levels were followed (Table 4).

ACh of the double mutants *p1152 cn347*, that is, a combination of low and high ACh, was kept at high levels but lower than in the single *cn347* mutation. ACh levels were lower than expected in the double mutation *e245 cn257*, that is, the combination of normal and high ACh levels in the respective single mutation. In other double mutations, ACh levels are higher than additive levels of the single mutation. High ACh levels were maintained in the double mutants *e113 cn347, cn252 cn4146, cn252 cn257* and *cn490 cn4146* that were a combination of normal and high levels of ACh. The ACh levels in the double mutants *e245 cn490, e245 cn252, cn347 cn252* and *cn252 cn490* were much higher than in a single mutation.

DISCUSSION

To aid in understanding, the phenotypes of the six mutants mentioned above are summarized in Table 5. The six trichlorfon resistants all showed a similar kinky paralysis but were classified into two groups based on the other two phenotypes, development and ACh levels. In one group, the *unc-10* and the *unc-3* gene mutations were normal but in the other group, the *unc-13, unc-18, unc-41* and *unc-17* gene mutations were abnormal in de-

Table 4. ACh levels of double mutants of trichlorfon resistance

Genotype	Ach levels
	nmol/ mg protein
dpy-13(*e184*)	0.154±0.052
cha-1(*p1152*) *dpy-13*(*e184*) *unc-18*(*cn347*)	0.502±0.032
unc-17(*cn113*) *dpy-13*(*e184*) *unc-18*(*cn347*)	0.733±0.046
unc-17(*cn245*) *dpy-13*(*e184*) *unc-13*(*cn490*)	1.77 ±0.055
unc-17(*cn245*) *dpy-13*(*e184*) *unc-41*(*cn252*)	1.81 ±0.145
unc-17(*cn245*) *dpy-13*(*e184*) *unc-10*(*cn257*)	0.347±0.038
unc-17(*cn245*) *dpy-13*(*e184*) *unc-3*(*cn4146*)	0.777±0.059
dpy-11(*e224*)	0.187±0.024
unc-41(*cn252*) *dpy-11*(*e224*) *unc-18*(*cn347*)	1.48 ±0.008
unc-41(*cn252*) *dpy-11*(*e224*) *unc-13*(*cn490*)	1.46 ±0.176
unc-41(*cn252*) *dpy-11*(*e61*) *unc-3*(*cn4146*)	0.681±0.008
unc-41(*cn252*) *dpy-11*(*e224*) *unc-10*(*cn257*)	0.632±0.023
dpy-5(*e61*)	0.149±0.042
unc-13(*cn490*) *dpy-5*(*e61*) *unc-3*(*cn4146*)	0.804±0.053
unc-13(*cn490*) *dpy-5*(*e61*) *unc-10*(*cn257*)	0.896±0.015

Table 5. Summary of phenotypes of six mutants

Matations	Development	Bahavior	Sensitivity to AChE inhibitors	ChAT activity	Choline levels	ACh levels
unc-10(*cn257*), *unc-3*(*cn4146*)	normal	kinky paralysis	resistant	normal	normal	normal
unc-13(*cn490*), *unc-41*(*cn252*), *unc-18*(*cn347*), *unc-17*(*cn355*)	slow growth, small and thin body	kinky paralysis	resistant	normal	normal	high

velopment and ACh levels. We are especially interested in mutations causing abnormal accumulation of ACh. Information on the localization of ACh at the synaptic level was scarce in *C. elegans*. Higher levels of ACh were also observed in the mutation of the *ace-2* gene that encodes one of three types of AChE [10]. However, ACh in this mutant may be accumulated at the synaptic gap because of a partial defect in ACh hydrolysis. This possibility is supported by the finding that the *ace-2* mutant is more sensitive to AChE inhibitors than wild-type animals. In contrast to the *ace-2* mutant, the *unc-17*(*e245*) mutant was resistant to AChE inhibitors and the ACh levels were no longer influenced by the addition of reagents. From these results, it is hypothesized that ACh in the mutant is not released into the synaptic gap [10]. From the similarity between the phenotypes of *unc-17*(*e245*) and *unc-13, unc-41,* and *unc-18* gene mutants, the functions of these genes might be partially overlapping. Indeed, ACh levels were higher in the four double mutants between these genes than in the respective single mutants. Therefore, ACh in the nematode might be accumulated by multiple pathways rather than by a single pathway. However, these pathways are not independent but might be interactive because the ACh levels in double mutants are much higher than additive levels. A major goal of our work is to elucidate the genetic basis of the synaptic transmission. Some insight into the molecular nature of the gene products may be very useful for accomplishing this. We have started and recently cloned the 6.8 kb BglII DNA fragment including the *unc-18* gene (Fig. 3) into the plasmid pUC 18 (unpubl. results).

Morphological defects in neurons have been revealed in the *unc-13* and *unc-3* gene mutants. In the *unc-13* mutations, some interneurons in the ventral cord had extraneous gap junctions to motorneurons in addition to normal neural connections (I. Maruyama, personal communication). In the *unc-3* mutations, the processes of the interneurons are disorganized along the cord leading to wrong synaptic inputs from the interneurons [30, 31]. It is probable that mutations showing abnormal accumulation of ACh are also defective in the function of motor neurons because about three-quarters of the neurones in the ventral nerve cord are occupied by cholinergic neurones in nematodes [5]. We cannot infer how the response to trichlorfon is controlled in the nematode because of limitations in the knowledge about the neuroanatomy or molecular defects of the mutant strains. It is possible that the response of these mutations to trichlorfon may be an indirect consequence of grosser cellular or subcellular defects. From preliminary work, we found that in doubles constructed with *cha-1, unc-18, unc-17, unc-13, unc-41, unc-3,* and *unc-10* mutations, the one with the phenotype of stronger trichlorfon resistance of the two was expressed in many cases, though the extent of the resistant was variable. However, any combination of *unc-17, unc-10* and *unc-13* mutations was no longer resistance to trichlorfon, suggesting the functional interactions of these genes.

We began this work with the hope of isolating spontaneous mutants of the *cha-1·unc-17* complex gene induced by the insertion of Tcl. One allele of the *unc-17* region but no alleles of the *cha-1* region were isolated through this work. Recently, Dr. J. Rand also isolated spontaneous resistant mutants to the AChE inhibitor, aldicarb and found alleles of *unc-17* but no alleles of *cha-1*. One reason why

no spontaneous *cha-1* mutant can be found is that the site for the Tcl insertion may be defective in the *cha-1* region. The frequencies of spontaneous Tcl-induced mutations are not even throughout the chromosomal regions but greatly varied, e.g., the frequencies for the *unc-54, lin-12* and *unc-22* genes are 5×10^{-7}, 5×10^{-5}, 1×10^{-4}, respectively (see review by Herman and Shaw [11]). The other possibility is that the mutant induced by Tcl insertion at the *cha-1* region is either lethal or the growth is too slow to detect in the screening. No mull allele in the *C. elegans cha-1* gene has so far been isolated [9, 22]. Totally ChAT defective mutants may be lethal as seen in *Drosophila cha* mutants [32]. It may also be possible that the growth of the *cha-1* mutants induced by Tcl insertion is so greatly affected that the mutants could not be detected from the screening plates. The screening method for trichlorfon resistans described here is not complete because most animals can recover from the inhibition when transferred under highly condensed states on the screening plates.

In this report, we presented a genetic analyses of six genes showing resistance to trichlorfon. The eleven remaining trichlorfon resistants isolated in this work were accompanied by marginal uncoordinated phenotypes and complemented the six gene mutants, indicating the presence of other genes resistant to AChE inhibitors in addition to the six described. We cannot predict how many genes affect the worm's response to AChE inhibitors. From the preliminary screening of uncoordinated mutants isolated in addition to the six genes, seven gene mutants were found to be resistant to AChE inhibitors: *unc-63, unc-11, unc-32, unc-36, unc-64, unc-65* and *unc-1* (J. M. RAND, personal communication). The mutations in the *unc-63, unc-11, unc-32, unc-36, unc-64, unc-65* and *unc-1* genes were also classified into two groups based on the body size of the mature animals: nearly normal (*unc-32, unc-36,* and *unc-65*) and small (*unc-63, unc-64, unc-11,* and *unc-1*). The latter group except for *unc-1* developed slowly. The *unc-1(e94)* mutation resulted in a small body but with normal development. Measurement of ACh levels in these mutants is in progress.

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REFERENCES

1 Reichard, L. F. and Kelly, R. B. (1983) A molecular description of nerve terminal function. Annu. Rev. Biochem., **52**: 871–926.

2 Miyamoto, M. D. (1978) The actions of cholinergic drugs on motor nerve terminals. Pharmacol. Rev., **29**: 221–247.

3 Tůcek, S. (1985) Regulation of acetylcholine synthesis in the brain. J. Neurochem., **44**: 11–24.

4 Brenner, S. (1974) The genetics of *Caenorhabditis elegans*. Genetics, **77**: 71–94.

5 Johnson, C. D. and Stretton, A. O. W. (1980) Neural control of locomotion in *Ascaris*: Anatomy, electrophysiology and biochemistry. *1* In "*Nematodes as biological models*". Ed. by B. Zuckerman, Academic Press, New York/ London, pp. 159–195.

6 White, J. G., Southgate, E., Thomson, J. N. and Brenner, S. (1986) The structure of the nervous system of the nematode *Caenorhabditis elegans*. Phil. Trans. R. Soc. Lond. B, **314**: 1–340.

7 Rand, J. B. and Russell, R. L. (1984) Choline acetyltransferase-deficient mutants of the nematode *Caenorhabditis elegans*. Genetics, **106**: 227–248.

8 Hosono, R., Kuno, S. and Midsukami, M. (1985) Temperature-sensitive mutation causing reversible paralysis in *Caenorhabditis elegans*. J. Exp. Zool., **235**: 409–421.

9 Sassa, T., Hosono, R. and Kuno, S. (1987) Choline acetyltransferase from a temperature-sensitive mutant of *C. elegans*. Neurochem. Int., **11**: 323–329.

10 Hosono, R., Sassa, T. and Kuno, S. (1987) Mutations affecting acetylcholine levels in the nematode *Caenorhabditis elegans*. J. Neurochem., **49**: 1820–1823.

11 Herman, R. K. and Shaw, J. E. (1987) The transposable genetic element Tcl in the nematode *Caenorhabditis elegans*. Trends Genet., **3**: 222–225.

12 Greenwald, I. (1985) *lin-12*, a nematode homeotic gene, is homologous to a set of mammalian proteins that includes epidermal growth factor. Cell, **43**: 583–590.

13 Moerman, D. G., Benian, G. M. and Waterston, R. H. (1986) Molecular cloning of the muscle gene

unc-22 in *Caenorhabditis elegans* by Tcl transposon tagging. Proc. Natl. Acad. Sci. USA, **83**: 2579–2583.

14 Rosenzweig, B., Liao, L. W. and Hirsh, D. (1983) Sequence of the *C. elegans* transposable element Tcl. Nucleic Acids Res., **11**: 4201–4209.

15 Rosenzweig, B., Liao, L. W. and Hirsh, D. (1983) Target sequences for the *C. elegans* transposable element Tcl. Nucleic Acids Res., **11**: 7137–7140.

16 Emmons, S. W., Yesner, L., Ruan, K. and Kastzenberg, D. (1983) Evidence for a transposon in *Caenorhabditis elegans*. Cell, **32**: 55–65.

17 Liao, L. W., Rosenzweig, B. and Hirsh, D. (1983) Analysis of a transposable element in *Caenorhabditis elegans*. Proc. Natl. Acad. Sci. USA, **80**: 3585–3589.

18 Moerman, D. G. and Waterston, R. H. (1984) Spontaneous unstable *unc-22* IV mutations in *C. elegans* var. Bergerac. Genetics, **108**: 859–877.

19 Eide, D. and Anderson, P. (1985) The gene structures of spontaneous mutations affecting a *Caenorhabditis elegans* myosin heavy chain gene. Genetics, **109**: 67–79.

20 Eide, D. and Anderson, P. (1985) Transposition of Tcl in the nematode *Caenorhabditis elegans*. Proc. Natl. Acad. Sci. USA, **82**: 1756–1760.

21 Horvitz, H. R., Brenner, S., Hodgkin, J. and Herman, R. K. (1979) A uniform genetic nomenclature for the nematode *Caenorhabditis elegans*. Mol. Gen. Genet., **175**: 129–133.

22 Rand, J. B. and Russell, R. L. (1985) Properties and partial purification of choline acetyltransferase from the nematode *Caenorhabditis elegans*. J. Neurochem., **44**: 189–200.

23 McCaman, R. E. and Stetzler, J. (1977) Radiochemical assay for ACh: modifications for sub-picomole measurements. J. Neurochem., **28**: 669–671.

24 Sulston, J. and Brenner, S. (1974) The DNA of *Caenorhabditis elegans* Genetics, **77**: 95–104.

25 Emmons, S. W. and Yesner, L. (1984) High-frequency excision of transposable element Tcl in the nematode *Caenorhabditis elegans* is limited to somatic cells. Cell, **36**: 599–605.

26 Southern, E. M (1975) Detection of specific sequences among DNA fragments by gel electrophoresis. J. Mol. Biol., **98**: 503–517.

27 Rigby, P. W. J., Dieckmann, M., Rhodes, C. and Berg, P. (1977) Labeling deoxyribonucleic acid to high specific activity in vitro by nick translation with DNA polymerase 1. J. Mol. Biol., **113**: 237–251.

28 Hodgkin, J. (1983) Male phenotypes and mating efficiency in *Caenorhabditis elegans*. Genetics, **103**: 43–64.

29 Johnson, C. D. and Russell, R. L. (1983) Multiple molecular forms of acetylcholinesterase in the nematode *Caenorhabditis elegans*. J. Neuronchem., **41**: 30–46.

30 Herman, R. K. (1984) Analysis of genetic mosaics of the nematode *Caenorhabditis elegans*. Genetics, **108**: 165–180.

31 Herman, R. K. (1987) Mosaic analysis of two genes that affect nervous system structure in *Caenorhabditis elegans*. Genetics, **116**: 377–388.

32 Greenspan, R. J. (1980) Mutations of choline acetyltransferase and associated neural defects in *Drosophila melanogaster*. J. Comp. Physiol., **137**: 83–92.